PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Michael A. ZASLOFF *et al.*	
Application No.: 09/985,417	Group Art Unit: 1616
Filed: November 2, 2001	Examiner: Sabiha N. Qazi, Ph.D.
For: AMINOSTEROL COMPOUNDS USEFUL AS INHIBITORS OF THE SODIUM/ PROTON EXCHANGER (NHE), PHARMACEUTICAL METHODS AND COMPOSITIONS EMPLOYING SUCH INHIBITORS, AND PROCESSES FOR EVALUATING THE NHE-INHIBITORY EFFICACY OF COMPOUNDS	

DECLARATION OF KENNETH J. HOLROYD, M.D. UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
United States Patent & Trademark Office
Washington, DC 20231

Sir:

I, Kenneth J. Holroyd, M.D. declare that:

1. In the context of my employment with Genaera, the assignee of the subject application, I was asked to conduct the primate studies referenced below.

2. I received my medical degree in 1984 at the Johns Hopkins University School of Medicine. I am considered an expert in my field with over 18 years of experience in the field of internal medicine, including 18 years working with matters relating to ophthalmology. My CV is attached as Exhibit A.

3. Iris and corneal neovascularization are symptoms of retinopathy. Tests conducted on iris neovascularization are accepted in the field as verifiable tests and accepted models for retinopathy, including use for identification of effective treatments for retinopathy, including diabetic retinopathy. A document from the Handbook of Ocular Disease Management discussing diabetic retinopathy is attached as Exhibit B.

4. Wet macular degeneration is a specific type of abnormal ocular neovascularization. As such, tests conducted on iris neovascularization are also accepted in the field as verifiable tests and accepted models for wet macular degeneration, including use for identification of effective treatments for wet macular degeneration. A document from the Handbook of Ocular Disease Management discussing macular degeneration is attached as Exhibit C.

5. Tests were conducted under my direction and control to determine if squalamine could inhibit iris neovascularization in cynomolgus monkeys. In this study, experimental iris neovascularization was induced into one eye of each cynomolgus monkey through vein occlusion. The eyes were evaluated approximately every three days for iris neovascularization by slit lamp examination and using fluorescein angiography. The iris neovascularization was graded using the scale published by Miller et al. (attached as Exhibit D) and reproduced below in Table 1. All animals were sacrificed at the end of the treatment.

Table 1: Iris Neovascularization Grading Scale

Grade	Description
0	Vessels may or may not be visible, depending on the degree of brown iris pigmentation. On angiography, the vessels fill briefly with fluorescein, are radial, and do not leak any fluorescein. Grade 0 is an example of a normal, healthy iris.
1	The vessels appear more prominent and tortuous than in the normal state, appear discontinuous, but do not leak fluorescein.
2	The vessels are more prominent, are nonradial, and leak fluorescein late in the angiogram.
3	The vessels are more prominent, are nonradial, and leak fluorescein early in the angiogram (within 20 to 30 seconds).
4	Individual vessels cannot be delineated in the early frames of the angiogram (within 20 to 30 seconds), and the iris appears as a diffuse opaque, fluorescent sheet.
5	The grade is angiographically identical to grade 4; however, it is associated with hyphema and ectropion uveae.

6. In one study (test 1), squalamine was administered (1 mg/kg squalamine dissolved in 100 mL of 5% dextrose in water) by continuous intravenous infusion to four monkeys. Four other monkeys received a placebo (100 mL of 5% dextrose in water) and were used as a control. The infusions were administered via an infusion pump set for a period of one hour. Infusions began immediately after vein occlusion and were repeated twice weekly for the next two weeks.

7. In a second study (test 2), squalamine was administered (1 mg/kg squalamine dissolved in 100 mL of 5% dextrose in water) by continuous intravenous infusion to four monkeys. Two other monkeys received a placebo (100 mL of 5% dextrose in water) and were used as a control. The infusions were administered via an infusion pump set for a period of one hour. Infusions began on day 7 (after the development of iris neovascularization) and were repeated twice weekly for the next two weeks.

8. The results of test 1 are reported below in Table 2. Table 2 illustrates the difference between the eyes of monkeys that are treated with squalamine while induced with experimental iris neovascularization and the eyes of monkeys treated with a placebo while induced with experimental iris neovascularization. The control monkeys all developed extensive iris neovascularization within nine days after vein occlusion (three developed grade 4 and one developed grade 5 iris neovascularization). In contrast, moneys treated with squalamine all developed only a mild form of iris neovascularization (grade 1 or 2) seven days after vein occlusion. Fourteen days after treatment with squalamine, two of the four eyes in the squalamine-treated monkey group exhibited no clinical signs of iris neovascularization (grade 0), and two others retained only a mild form of iris neovascularization (grade 2).

Table 2: Grading of Iris Neovascularization in Test 1

Monkey	Time after vein occlusion						
	4 days	7 days	9 days	14 days	17 days	21 days	23 days
1-control	0	4	4	4	4	4	4
2-control	0	3	4	4	4	4	4
3-control	0	5	5	5	5	5	5
4-control	0	4	4	4	4	4	4
5-drug	0	2	2	2	2	2	2
6-drug	0	1	1	2	2	2	2
7-drug	0	1	1	0	0	0	0
8-drug	0	1	1	0	0	0	0

9. The results of test 2 are reported below in Table 3. Table 3 illustrates the difference between the eyes of monkeys that are treated with squalamine after experimental iris neovascularization has been induced and the eyes of monkeys treated with a placebo after

experimental iris neovascularization has been induced. After vein occlusion, all monkeys developed grade 4 iris neovascularization. The control monkeys, which were treated with placebo, all retained the extensive levels of iris neovascularization (grade 4) throughout the placebo-treatment period. In contrast, moneys treated with squalamine all showed significant improvement upon the implementation of the squalamine treatment. Seven days after treatment with squalamine was initiated, two of the eyes in squalamine-treated monkey group completely recovered, showing no clinical signs of iris neovascularization (grade 0). The other two eyes improved from a severe case of iris neovascularization (grade 4) to only a mild form of iris neovascularization (grade 2).

Table 3: Grading of Iris Neovascularization in Test 2

Monkey	Grade of iris neovascularization 7 days after vein occlusion	Grade of iris neovascularization during the time after the development of iris neovascularization			
		4 days	7 days	11 days	15 days
1-control	4	4	4	4	4
2-control	4	4	4	4	4
3-drug	4	4	2	2	2
4-drug	4	3	2	2	2
5-drug	4	3	0	0	0
6-drug	4	2	0	0	0

10. I conclude that the above studies demonstrate that squalamine will effectively treat iris neovascularization, and, consequently, will effectively treat retinopathy.

11. In the subject patent application, it was demonstrated that squalamine, compounds 319, 353 and 410 inhibit angiogenesis to the same or greater degree than fumagillin, a standard anti-angiogenic. See Table 3, page 98 of the specification. It was also demonstrated in the subject application that squalamine, compounds 319 and 415 exhibited the greatest activity with regard to vascular regression and NHE inhibition. See Table 5 on page 104 of the specification. Based on the neovasculative-inhibitory effects demonstrated by squalamine, compounds 353, 415, 410 and 319, I conclude that compounds 353, 415, 410 and 319 would effectively treat retinopathy and produce the same or similar results in the above-referenced squalamine studies.

12. I conclude that the above studies demonstrate that squalamine will effectively treat iris neovascularization, and, consequently, will effectively treat wet macular degeneration.

13. In the subject patent application, it was demonstrated that squalamine, compounds 319, 353 and 410 inhibit angiogenesis to the same or greater degree than fumagillin, a standard antiangiogenic. See Table 3, page 98 of the specification. It was also demonstrated in the subject application that squalamine, compounds 319 and 415 exhibited the greatest activity with regard to vascular regression and NHE inhibition. See Table 5 on page 104 of the specification. Based on the neovasculative-inhibitory effects demonstrated by squalamine, compounds 353, 415, 410 and 319, I conclude that compounds 353, 415, 410 and 319 would effectively treat wet macular degeneration and produce the same or similar results in the above-referenced squalamine studies.

14. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are so punishable by fine or imprisonment, or both under 18 U.S.C § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Kenneth J. Holroyd

Kenneth J. Holroyd, M.D.

April 10, 2002

Date

Attached:

Exhibit A: CV of Kenneth J. Holroyd, M.D.

Exhibit B: Diabetic Retinopathy, produced from the website www.revoptom.com in the section entitled "Handbook of Ocular Disease Management."

Exhibit C: Macular Degeneration, produced from the website www.revoptom.com in the section entitled "Handbook of Ocular Disease Management."

Exhibit D: Miller J.W. et al., *Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model*, 145 American Journal of Pathology 574-84 (1994).

Curriculum Vitae

Name: Kenneth James Holroyd

Education:
B.A., Johns Hopkins University, Natural Science, 1980
M.D., Johns Hopkins University School of Medicine, 1984
M.B.A., Johns Hopkins University, 2000

Board Certification:
FLEX Medical Licensure Examination, 1986
Internal Medicine, American Board of Internal Medicine, 1987
Pulmonary Medicine, American Board of Internal Medicine, 1990
Anesthesiology, American Board of Anesthesiology, 1995

Employment:
1984-1987 Intern and Resident, Department of Internal Medicine, Johns Hopkins Hospital and School of Medicine
1987-1988 Instructor in Medicine and Assistant Chief of Service, Department of Internal Medicine, Johns Hopkins Hospital and School of Medicine
1988-1991 Senior Staff Fellow, Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health
1990-1991 Senior Clinical Staff, Clinical Center, National Institutes of Health
1991-1993 Resident and Fellow, Anesthesiology and Critical Care Medicine, Johns Hopkins Hospital and School of Medicine
1994-1997 Assistant Professor, Anesthesiology and Critical Care Medicine, Johns Hopkins Hospital and School of Medicine
1996-1997 Joint Appointment as Assistant Professor, Pulmonary Medicine, Department of Internal Medicine, Johns Hopkins Hospital and School of Medicine
1996-1997 Medical Director, Respiratory Therapy and Respiratory Care Services, Johns Hopkins Hospital
1997-1998 Vice President, Respiratory Discovery Research and Product Development, Genaera Corporation
1997-1998 Vice President, Business Development, Genaera Corporation
1998-2000 Senior Vice President, Clinical Research and Regulatory Affairs, Genaera Corporation
2000-2002 Executive Vice President and Chief Business Officer, Genaera Corporation
2002- now Executive Vice President and Chief Operating Officer, Genaera Corporation

Publications:

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HANDBOOK of Ocular Disease Management

DIABETIC RETINOPATHY

SIGNS AND SYMPTOMS

A microvascular disease that primarily affects the capillaries, diabetes mellitus affects the eye by destroying the vasculature in the conjunctiva, retina and central nervous system. Patients may present with histories of long-standing injected bulbar conjunctivae along with systemic complaints of weight loss despite larger than normal appetite (polyphasia), abnormal thirst (polydypsia) and abnormally frequent urination (polyuria).

Fluctuating visual acuity secondary to unstable blood sugar is a common ocular sign. Swelling within the crystalline lens results in large sudden shifts in refraction as well as premature cataract formation. Changes in visual acuity will depend upon the severity and stage of the disease.

In the retina, weakening of the arterioles and capillaries may result in the characteristic appearance of intraretinal dot and blot hemorrhages, exudates, intraretinal microvascular abnormalities (IRMA) microaneurysms, edema and cotton wool infarcts. Proliferative diabetic retinopathy is the result of severe vascular compromise and is visible as neovascularization of the disc (NVD), neovascularization elsewhere (NVE) and neovascularization of the iris (NVI, or rubeosis irides). Neurological complications include palsies of the third, fourth and sixth cranial nerves as well as diabetic papillitis and facial nerve paralysis.

PATHOPHYSIOLOGY

Diabetes mellitus is a genetically influenced group of diseases that share glucose intolerance. It is characterized as a disorder of metabolic regulation as a result of deficient or malfunctioning insulin or deficient or malfunctioning cellular insulin receptors.

Biochemistry involving the formation of sorbitol plays a role in the destruction of pericytes, which are cells that support the vascular endothelium. As the supportive pericytes perish, capillary endothelium becomes compromised, resulting in the vascular leakage of blood, protein and lipid. This, in combination with thickened, glucose-laden blood, produces vascular insufficiency, capillary nonperfusion, retinal hypoxia, altered structure and decreased function. The formation and release of vasoproliferative factors which play a role in the genesis of retinal neovascularization are poorly understood.

MANAGEMENT

When you suspect ocular sequelae of diabetes mellitus in an undiagnosed individual, either you or the patient's physician should order a fasting blood glucose (FBS), glycosylated hemoglobin or oral glucose tolerance test (OGTT).

Most non-vision threatening sequelae of diabetes resolve spontaneously over the course of weeks to months following medical control. In cases where there are large refractive changes, patients may require a temporary spectacle prescription until the refraction stabilizes. The most important element of

MANAGEMENT is education so that patients are informed that they may eventually need to change their spectacle lenses.

When retinopathy threatens the macula or when new blood vessels proliferate, refer for laser photocoagulation. The Diabetic Retinopathy Study (DRS) has conclusively proven that panretinal photocoagulation was successful in reducing the risk of severe vision loss in high risk patients. It defined the high risk characteristics as: (1) Neovascularization of the optic disc (NVD) one-quarter to one-third of a disc diameter in size and (2) Neovascularization elsewhere (NVE) with any vitreous hemorrhage.

If the patient exhibits either of these high risk characteristics, refer him or her to a vitreoretinal specialist.

The Early Treatment of Diabetic Retinopathy Study (ETDRS) has shown that focal/grid laser photocoagulation reduced the risk of moderate vision loss in patients with clinically significant macular edema, defined as: (1) retinal thickening at or within 500 microns (one-third of a disc diameter) of the center of the foveola, (2) exudate at or within 500 microns of the center of the foveola only if associated with retinal thickening, or (3) an area of retinal thickening one disc diameter or greater in size, within one disc diameter of the foveola.

If you observe any of these signs, regardless of the acuity, refer the patient to a retinal specialist. Referral is also indicated if

you suspect clinically significant macular edema but are having difficulty visualizing the macula or edema.

CLINICAL PEARLS

- Clinically significant macular edema is unrelated to acuity and can exist in the presence of 20/20 vision. It can only be identified through observation using stereoscopic indirect biomicroscopy (60D, 78D, 90D Hruby or three-mirror lens).

- Fluorescein angiography is only used for treatment. It identifies the areas of leakage that require focal grid laser photocoagulation. With respect to diabetes, it is not a tool for diagnosis. Angiography is not required for treating proliferative disease since PRP does not require precise aiming of the laser.

- The development of diabetic retinopathy is time-dependent. Even in the face of optimal blood sugar control, patients with long-standing disease can be expected to eventually develop some form of retinopathy.

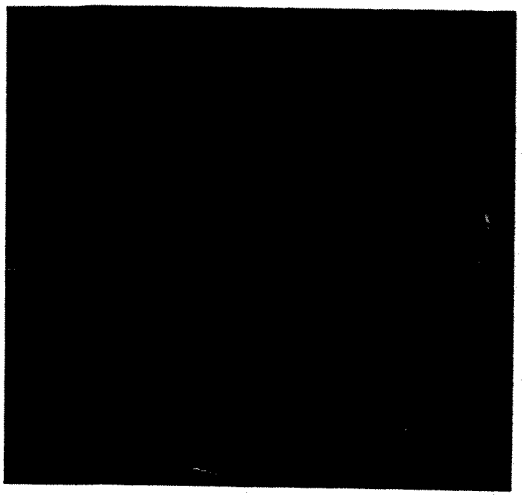
Other reports in this section

- Posterior Vitreous Detachment
- Tractional Retinal Tears
- Epiretinal Membrane
- Diabetic Retinopathy
- Lattice Degeneration
- Retinal Vein Occlusion
- Retinal Artery Occlusion
- Macular Degeneration
- Toxoplasmosis
- Choroidal Melanoma
- Acquired Retinoschisis
- Hollenhorst Plaque
- Idiopathic Central Serous Chorioretinopathy
- Macular Hole
- Presumed Ocular Histoplasmosis Syndrome
- Retinal Macroaneursym
- Retinitis Pigmentosa
- Retinal Detachment
- Hypertensive Retinopathy
- Retinal Pigment Epithelium (RPE) Detachment
- Papillophlebitis
- Ocular Ischemic Syndrome
- Coats' Disease and Leber's Miliary Aneurysm

Eyelids & Eyelashes | Conjunctiva & Sclera | Cornea
Uvea | Vitreous & Retina | Optic Nerve & Brain | Oculosystemic Disease
Handbook Main Page

HANDBOOK of Ocular Disease Management

MACULAR DEGENERATION



SIGNS AND SYMPTOMS

Age-related macular degeneration (AMD) is the leading cause of legal blindness in the United States of America for persons over the age of 65. AMD is present in approximately 10 percent of the population over the age of 52 and in up to 33 percent of individuals older than 75. AMD is an extension of abnormalities that begin and progress through Bruch's membrane, involving the retinal pigment epithelium (RPE) and photoreceptors. The earliest clinical manifestation of AMD are drusen and macular pigmentary atrophy. The presence of drusen does not indicate AMD. However, it serves as precursor, warning that there is the potential for progression and visual loss. AMD is bilateral in 55 percent of cases.

The visual symptoms associated with AMD depend on its severity and type. In general, the "dry" form (no subretinal choriodal neovascularization, exudation or hemorrhage) is less severe, producing a gradual, painless distortion or loss of central vision. Some patients complain of color distortion. "Wet" AMD (subretinal choriodal neovascularization, exudation and or hemorrhage) often produces severe central visual loss. Visual loss produced by wet AMD is often rapidly progressive.

Some of the clinical retinal signs of dry AMD include drusen of the posterior pole, granular clumping and disorganization of the RPE in the macular area, macular RPE hyperplasia and degeneration of the outer retinal layers with circumscribed areas of geographic atrophy of the RPE.

Some of the clinical retinal signs associated with wet AMD include hard and soft drusen, subretinal thickening secondary to classic or occult choroidal neovascularization (producing a grayish-green subretinal hue), subretinal, intraretinal or vitreous hemorrhage, subretinal and intraretinal exudation with serosanguinous fluid accumulation and fibrovascular scar formation (disciform scarring).

PATHOPHYSIOLOGY

All forms of AMD possess initial, common changes within the macular RPE. While the mechanisms and processes are poorly understood, some postulate these changes are initiated by isolated regions of choriocapillaris vascular failure. These proceedings cause the RPE to degenerate, resulting in photoreceptor loss. As the photoreceptors disintegrate, the inner nuclear layer collapses and contacts Bruch's membrane, initializing the degeneration of the outer retinal layers. Some theorize that the mechanism of damage may be through ultraviolet radiation-induced oxidation and free radical formation within these structures.

Wet AMD results when the macular RPE/Bruch's barrier is compromised by new, weak and leaky blood vessels that grow upward into the retina from the choriocapillaris. These occult (poorly defined) or classic (more easily defined) subretinal choroidal neovascular membranes may leak serosanguinous fluid causing RPE detachment, sensory retinal detachment, subretinal or intraretinal bleeding or fibrovascular, disciform scarring.

MANAGEMENT

Begin managing patients with potential or diagnosed AMD by recognizing the associated risk factors and providing patient education. The disease is more common in individuals who have a family history of AMD, are of light complexion, in those who have a cardiovascular history, history of previous lung infection, hyperopia or decreased hand grip strength. It is typically more progressive in males. Smoking is a significant risk factor.

The management of patients with dry AMD begins with biannual eye examination, with dilated funduscopy. Home therapy, aimed at early detection, using a home Amsler grid may work to monitor the stability of suspicious or involved maculae. The elimination of potentially harmful ultraviolet light using UV coatings on spectacles and sunglasses may also reduce the risk of photochemical /oxidative damage to the retina for all patients.

Researchers have indicated that oral antioxidants like vitamins C and E and oral zinc may play a role in reducing retinal damage by terminating the chemical reactions initiated by free radicals, created by retinal metabolism. Multiple vitamins, oral zinc or products specifically designed for this purpose, such as Ocuvite and ICaps, are useful as a tool for slowing the

progression of AMD.

The treatment for symptomatic wet AMD begins with a referral to the vitreoretinal specialist for intravenous fluorescein angiography. The Macular Photocoagulation Study (MPS) examined the efficacy of treating subretinal, extrafoveal (200 to 2,500 µm from the center of the foveal avascular zone, juxtafoveal (1 to 200 µm from the center) and subfoveal choroidal neovascular membranes with laser photocoagulation. Laser photocoagulation reduces the risk of severe vision loss in patients with definable subretinal choroidal neovascular membranes.

In cases where hemorrhage and exudate obscures the angiogram or the neovascular membrane is poorly defined by fluorescein angiography, indocyanine green angiography may offer an additional modality to determine if treatment is feasible. Unfortunately, the recurrence rate following treatment is approximately 50 percent. Most recurrences develop within the first year, making a three-month follow up schedule critical. The efficacy and effectiveness of surgical procedures for the removal of subretinal neovascular membranes are currently under investigation.

In cases where bilateral central visual acuity has been lost, low vision and vision rehabilitation specialists may be able to offer training or optical devices which improve patients' quality of life. Macular translocation surgery has been performed with significant success and restored usable vision to patients with wet ARMD.

CLINICAL PEARLS

- In general, the risk to patients with dry AMD for progression to wet AMD, over any given five-year period, is approximately 14 to 20 percent.
- In patients who have already lost one eye to wet AMD, over the course of five years, the risk of developing wet stage disease in the fellow eye is 10 percent for patients whose fellow eye has neither large drusen or pigment clumps, 30 percent for fellow eyes containing either large drusen or pigment clumps and 50 percent for fellow eyes with both pigment clumps and large drusen present.

Other reports in this section

- Posterior Vitreous Detachment
- Tractional Retinal Tears
- Epiretinal Membrane
- Diabetic Retinopathy
- Lattice Degeneration
- Retinal Vein Occlusion
- Retinal Artery Occlusion
- Macular Degeneration
- Toxoplasmosis
- Choroidal Melanoma
- Acquired Retinoschisis
- Hollenhorst Plaque
- Idiopathic Central Serous Chorioretinopathy
- Macular Hole
- Presumed Ocular Histoplasmosis Syndrome
- Retinal Macroaneursym
- Retinitis Pigmentosa
- Retinal Detachment
- Hypertensive Retinopathy
- Retinal Pigment Epithelium (RPE) Detachment
- Papillophlebitis
- Ocular Ischemic Syndrome
- Coats' Disease and Leber's Miliary Aneurysm

Uvea | Vitreous & Retina | Optic Nerve & Brain | Oculosystemic Disease
Handbook Main Page

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Vascular Endothelial Growth Factor/Vascular Permeability Factor Is Temporally and Spatially Correlated with Ocular Angiogenesis in a Primate Model

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Ischemia often precedes neovascularization. In ocular neovascularization, such as occurs in diabetic retinopathy, a diffusible angiogenic factor has been postulated to be produced by ischemic retina and to lead to neovascularization of the retina, optic nerve, or iris. However, no angiogenic factor has been conclusively identified that satisfies this hypothesis. Vascular endothelial growth factor/vascular permeability factor, hereafter referred to as VEGF, is a likely candidate for an ocular angiogenic factor because it is a secreted mitogen, specific for endothelial cells, and is upregulated by hypoxia. We investigated the association of VEGF with the development of experimental iris neovascularization in the cynomolgus monkey. Following the production of retinal ischemia by laser occlusion of all branch retinal veins, VEGF was increased in the aqueous fluid, and the aqueous VEGF levels changed synchronously and proportionally with the severity of iris neovascularization. Northern analysis and in situ hybridization revealed that VEGF messenger RNA is upregulated in the ischemic retina. These observations support the hypothesis that ocular neovascularization is regulated by a diffusible factor and identify VEGF as a likely candidate for a retina-derived vascular permeability and angiogenesis factor in vivo. (Am J Pathol 1994, 145:574–584)

Angiogenesis, or the growth of new capillaries from existing blood vessels, occurs physiologically in ovulation and embryogenesis, and pathologically in ocular neovascularization (such as retinal vein occlusion and diabetic retinopathy), in rheumatoid arthritis, and in solid tumors. Insight into the mechanisms governing angiogenesis in one system are likely to apply to other angiogenic processes. Ocular neovascularization, at once visible and accessible, provides an ideal *in vivo* system in which to investigate the mechanisms that control angiogenesis.

Clinically, iris and retinal neovascularization are associated with areas of retinal capillary closure and ischemic retina, leading to the hypothesis that a factor diffusing from retinal ischemia is responsible for neovascularization.[1,2] Although angiogenic activity has been demonstrated from ocular tissues,[3,4] no specific angiogenic factor has been conclusively implicated in ocular angiogenic diseases. Vascular endothelial growth factor/vascular permeability factor (VEGF), a heparin-binding, dimeric protein, is an endothelial cell mitogen *in vitro*, and induces increased vascular permeability and angiogenesis *in vivo*.[5–9] Unlike basic fibroblast growth factor, another angiogenic factor found in the retina, VEGF is a secreted endothelial-selective mitogen.[8,10] In addition, VEGF has been demonstrated to be upregulated by hypoxia *in vitro*[11,12]

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and in solid tumors *in vivo*,[12–15] making it an appealing candidate for mediating ocular angiogenesis.

We have used a model of iris neovascularization in the nonhuman primate to investigate mechanisms that control angiogenesis. Laser photocoagulation was used to occlude the retinal veins, producing an ischemic retina similar to human clinical disease, and demonstrable by fundus fluorescein angiography. In a related model of retinal vein occlusion in the miniature pig, the affected retina has been demonstrated to be hypoxic using an O_2-sensitive microelectrode.[16] Normal primate iris has barely detectable vessels that do not leak fluorescein, although in inflamed eyes, mild leakage does occur. Four to seven days following laser vein occlusion, new iris vessels appear, characterized angiographically by rapid, diffuse fluorescein leakage from a vessel network.[17] Histological examination has confirmed the appearance of new iris vessels in this model, with thin-walled branching capillaries.[17] We have used this model to demonstrate a temporal and spatial association between VEGF and iris neovascularization.

Materials and Methods

Animal Procedures

All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology Resolution on the use of animals for research, and guidelines established for animal care at the Massachusetts Eye and Ear Infirmary. Cynomolgus monkeys (*Maccaca fascicularis*) were anesthetized for all procedures with an intramuscular injection of a mixture of ketamine, 20 mg/kg (Ketalar, Parke-Davis, Morris Plains, NJ), diazepam, 1 mg/kg (Elkins-Sinn Inc., Cherry Hill, NJ), and atropine sulfate, 0.125 mg/kg (Gensia Laboratories Ltd, Irvine, CA). Supplemental anesthesia of ketamine (10 mg/kg intramuscular) assured a stable plane of general anesthesia. Proparacaine hydrochloride (0.5%) topical anesthetic drops (Alcon, Humacao, Puerto Rico) were administered before placement of any lid speculae and for pneumotonometry. Pupils were dilated as needed with 2.5% phenylephrine and 0.8% tropicamide drops. Animals were placed in a comfortable restraint device to allow head positioning for photography and angiography. Deeply anesthetized animals were killed with an intravenous injection of Somnlethal (JA Webster, Sterling, MA), a pentabarbital-based euthanasia solution approved by the American Veterinary Medical Association.

Primate Model of Iris Neovascularization

We used a cynomolgus monkey model of iris neovascularization adapted from a previously published model,[18] which simulates human retinal vein occlusion. Dye yellow (577 nm) laser light (Coherent Lambda Plus, Palo Alto, CA) was used to occlude all branch retinal veins in the eyes of cynomolgus monkeys. Fundus photography and fluorescein angiography, using 0.1 cc/kg of 10% sodium fluorescein via saphenous vein, was performed after laser vein occlusion. Angiography revealed venous occlusion retinopathy, with venous dilation, dot and blot hemorrhages, and areas of capillary nonperfusion, a hallmark of ischemic retina. Iris photography and fluorescein angiography were similarly performed using an adapter[19] mounted in front of a Canon CF-60ZA fundus camera (Lake Success, Long Island, NY). Angiograms were performed in a standard manner to obtain approximately 1 frame per second during the initial phase of the angiogram, and with both eyes photographed within the first 35 seconds after injection. Photographs were taken up to 2 minutes after injection, which captured the majority of the fluorescein leakage in this model.

A standardized grading system for iris neovascularization in this model has been developed, using standard photographs and angiograms (Miller et al. manuscript in preparation). This system analyzes the vessel density and degree of fluorescein leakage. Briefly, iris neovascularization is graded from 0 to 5, with grade 2 representing the threshold for classification as neovascularization. Grade 0 is a normal iris with few vessels visible and no leakage. Grade 1 has an increased vascular pattern without leakage and is typical of regressed neovascularization. A standard photograph 2A separates grades 2 and 3, with increasing vessel density and more rapid, early leakage of fluorescein. Mild, slow, leakage classed as grade 2 can be seen with inflammation, but higher grades are more specific for neovascular tissue. Grade 4 has enough density of neovascular tissue that the iris appears opaque within the first 35 seconds of the angiogram. Grade 5 has the characteristics of grade 4 plus ectropion uveae or glaucoma. The photographs and angiograms were coded by a technician, masked as to their date and animal, and then graded by two independent investigators. Using the grading system, the correlation between the two independent readers was 0.92.

Aqueous samples were obtained from sedated animals, using a 30-gauge needle attached to a siliconized 1-cc syringe passed through the limbus. After removal of aqueous fluid, a drop of gentamicin

sulfate was instilled into the fornix. Vitreous samples were obtained only after enucleation. Aqueous and vitreous samples were frozen at −4 C until they were assayed for VEGF.

Association of VEGF Level and Iris Neovascularization

A pilot study was performed to identify any relationship between VEGF level and iris neovascularization. Laser vein occlusion was performed in four eyes of two monkeys in the first experiment, which followed Iris neovascularization grade (as described above) and aqueous VEGF levels for 35 to 37 days after laser. Fluorescein angiography was performed and aqueous samples were obtained every 6 days.

In a second set of experiments, a control was incorporated that simulated the laser injury without producing retinal ischemia. Four animals were used in which the right eye underwent laser vein occlusion and the left eye underwent "sham" laser, for a total of eight eyes. In the sham lasered eyes, laser spots were placed adjacent to the retinal veins without producing vein occlusion or retinal ischemia, using the same number of laser spots, with the same spot size, power, and duration. Thus each animal was its own control, with one ischemic eye, and one nonischemic eye. Fluorescein angiograms and VEGF sampling were performed every 2 to 4 days to characterize the changes as iris neovascularization developed. The animals were followed for 13 to 15 days after laser. One monkey suffered an anesthetic accident and died 5 days after laser, so that only six eyes were followed beyond 6 days.

VEGF Protein Assay

VEGF levels in the aqueous were measured using a two-site time-resolved immunofluorometric assay, originally developed to measure guinea pig VEGF,[20] and modified to measure human VEGF using homogeneous recombinant human VEGF as calibrators.[21] The antibodies were prepared against the N- and C-terminal peptides of VEGF.[9,22] It was assumed that there would be substantial cross-reactivity between the monkey and human VEGF. Subsequently, we have cloned and sequenced monkey VEGF and have found that although there are minor changes in the complementary (c)DNA sequence, the amino acid sequence is identical (Shima et al, manuscript in preparation). Antibody to the carboxy terminus of human VEGF was first immobilized on microtiter wells. Aliquots (50 µl) of samples to be assayed were incubated with gentle shaking overnight at 4 C with 50 µl of assay buffer in the wells. After washing the wells, 100 µl of assay buffer containing europium chelate-(Eu^{3+}) labeled antibody to the amino terminus of VEGF, was then added to the wells and incubated at room temperature for 2 hours. The wells were washed, an enhancement solution added, and fluorescence was measured in a time-resolved fluorometer. The Eu^{3+} chelate has a long decay time (>500 µs), which allows reading of fluorescence to be determined between 400 and 800 µsec in time-dependent fashion. Most endogenous nonspecific fluorescence occurs at shorter times, which improves the signal to noise ratio in this assay. The interassay coefficient of variation at 25 pM is 13.7%, and the minimal detection limit is 5.5 pM. Because both the biological samples and the calibrators are in an environment of assay buffer, there is little concern that VEGF detection in biological fluids such as aqueous would be poor. However, to confirm this, the VEGF immunoassay was performed on monkey aqueous to which a known quantity of VEGF was added, and the recovery was 94% (MW = 42 kd for VEGF calculations).

Statistical Methods

The VEGF level and iris grade were displayed as scatterplots. Each was plotted against time, and for the data from the second experiment, VEGF level was plotted against iris grade. Curves were drawn by connecting x-y medians, using cubic splines to approximate the x-y relationships.[23] Because each animal contributed both an ischemic and a nonischemic, sham measure of VEGF and iris grade at each time point, the measures were summarized as paired differences (ie, ischemic minus nonischemic value for each animal at each time point). This hac the effect of "correcting" the vein-occluded eye for the "background" VEGF level or iris grade that is due to the laser injury. Because the control was the second eye of the same animal, other sources of variability were minimized. We looked to see if these corrected measures increased or decreased over time and to see if the corrected VEGF level and iris grade increased or decreased together, using analysis of variance[24] at successive time points contributed by each animal. Both VEGF level and iris grade were ranked before the analysis of variance because they were not normally distributed.[25]

Northern Analysis

Retinas were isolated from anesthetized animals before sacrifice and immediately frozen in liquid N_2 for

storage. The tissue was rapidly thawed by placing it into 5-ml, room temperature guanidinium buffer. The tissue was solubilized with a polytron and total RNA was isolated from retina using the guanidinium isothiocyanate/CsCl method.[26] RNA (15 µg per sample) was fractionated by electrophoresis on a 1.0% agarose-formaldehyde gel. The samples were transferred to nylon filters by capillary blotting overnight, and ultraviolet cross-linked. The filter was prehybridized for 4 hours at 42 C with 6× sodium chloride phosphate ethylenediaminetetraacetic acid, 5× Denhardt's, 50% formamide, 0.5% sodium dodecyl sulfate, and 100 µg/ml salmon sperm DNA followed by an 18-hour incubation with a random prime-labeled, 520-bp, *NcoI/BglII* fragment of the cloned human VEGF cDNA, a gift provided by Dr. Herbert Weich. Filters were washed once with 1× sodium chloride phosphate ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate (37 C), followed by two 10-minute washes with 0.1× sodium chloride phosphate ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate at 65 C. The same blot was stripped and reprobed with a 280-bp fragment of bovine 28S recombinant RNA cDNA labeled by random priming. The bands were visualized by autoradiography for 48 hours at −80 C.

In Situ *Hybridization*

The *in situ* hybridization protocol and the preparation of ^{35}S-labeled VEGF riboprobes and control sense riboprobes have been previously described.[14,27,28] Briefly, ^{35}S-labeled, single-stranded anti-sense and sense RNA probes for VEGF messenger (m)RNA were prepared. The anti-sense probe hybridizes with a region of VEGF mRNA coding sequence common to all four of the known splice variants of VEGF. Eyes for *in situ* hybridization were fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4 C, and then transferred to 30% sucrose in phosphate-buffered saline, pH 7.4, overnight. Tissue was then embedded in paraffin, and 4-µ sections were hybridized overnight. The slides were washed with serial solutions before and after hybridization. Slides were then dehydrated, coated with emulsion, and stored. After a 2-week exposure, the emulsion was developed and the slides counterstained.

Results

Association of VEGF Levels with Severity of Iris Neovascularization

In a pilot experiment, four eyes of two monkeys were followed for 35 to 37 days after laser retinal vein occlusion. Fluorescein angiography was performed and aqueous samples were obtained every 6 days to measure the development of iris neovascularization and aqueous VEGF level. Figure 1, A and B, shows the relationship between aqueous VEGF levels and the grade of iris neovascularization in the four eyes, and time. There was substantial variability in the degree of iris neovascularization, and in the aqueous VEGF levels from monkey to monkey. However, it is apparent that both measures change over time, and there seems to be some temporal association between changing aqueous VEGF level and changing iris neovascularization. VEGF was undetectable in the aqueous before laser vein occlusion, rose above 30 pM as iris neovascularization developed, and then fell

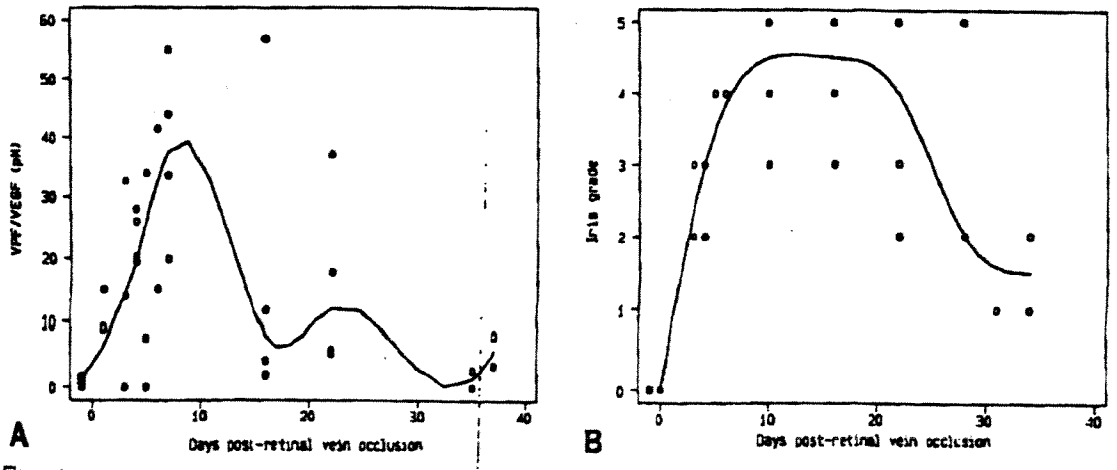

Figure 1. *Aqueous VEGF levels in eyes with iris neovascularization. A and B: VEGF levels in the aqueous (A) and grade of iris neovascularization (B) of four monkey eyes are compared over time after laser vein occlusion as a scatterplot with best fit curves. Although there is substantial variability between eyes, there seems to be an association between VEGF level and iris neovascularization. VEGF was undetectable in the aqueous before laser, the levels rise as neovascularization develops, and fall as the neovascularization regresses.*

to less than 10 pM as new vessels regressed. The level of VEGF detected in the aqueous as iris neovascularization developed was well above the concentration required for maximal stimulation of vascular endothelial cells *in vitro*. VEGF was undetectable in serum throughout the course of the experiment, making it unlikely that the VEGF measured in the aqueous was derived from the blood.

A second set of experiments was undertaken that incorporated a sham laser control to verify that increased aqueous VEGF and iris neovascularization was not due to a nonspecific response to retinal laser injury. In the sham eyes, or nonischemic eyes, laser spots were placed adjacent to the retinal veins without producing vein occlusion or retinal ischemia. The fundus appearance of an ischemic and a nonischemic eye is shown in Figure 2, A and D. Fluorescein angiography of an eye with laser vein occlusion demonstrated venous occlusion and ischemic retinopathy, with retinal capillary closure in the distribution of

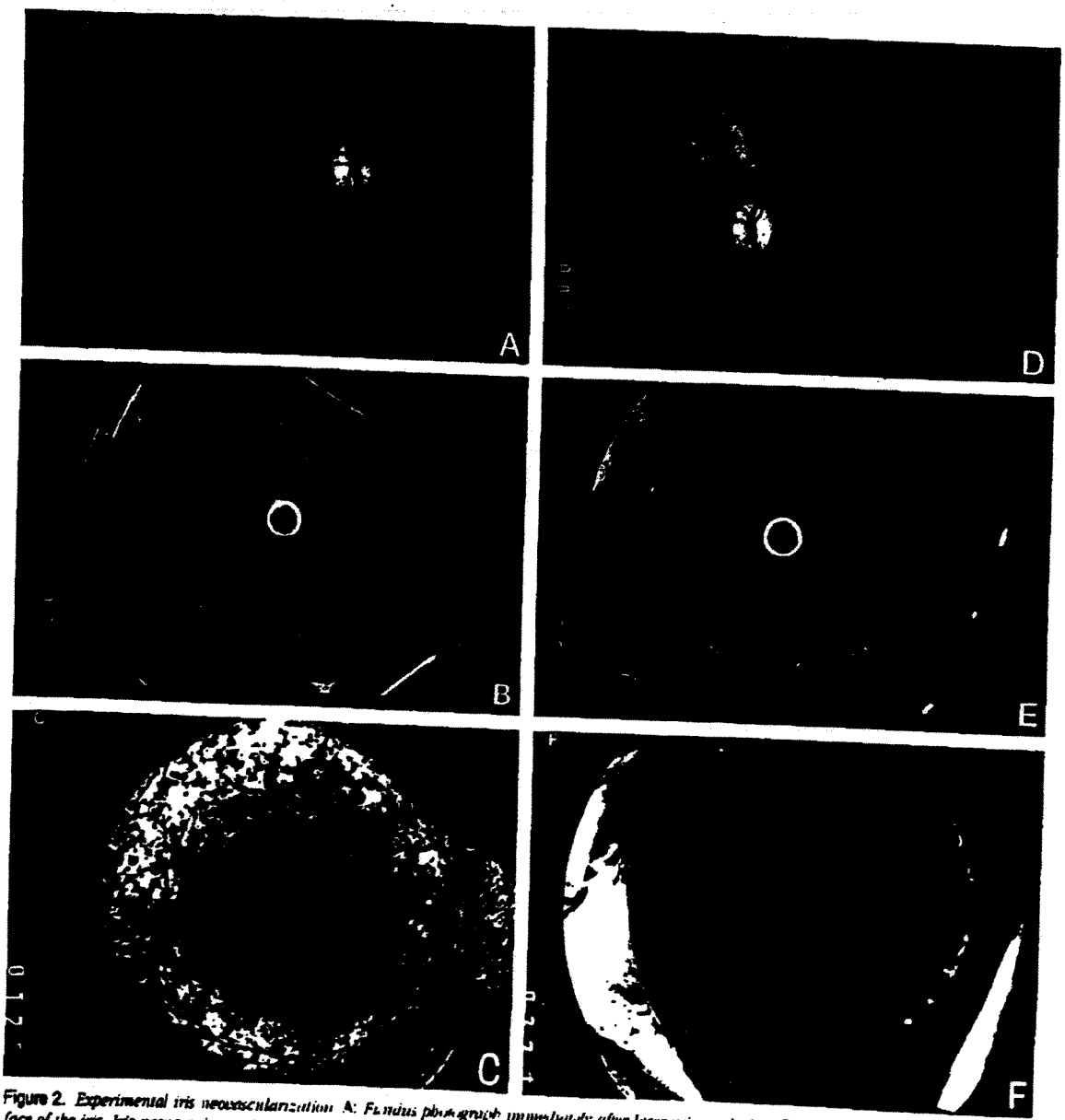

Figure 2. *Experimental iris neovascularization.* A: *Fundus photograph immediately after laser vein occlusion.* B: *New vessels are seen on the surface of the iris. Iris neovascularization appears* [illegible] *7 days after laser vein occlusion, increases, and persists for an average of 24 days, or leads to the development of neovascular glaucoma.* C: *Fluorescein angiography demonstrates new vessels on the surface of the iris, with a characteristic tortuous pattern, and abundant leakage of fluorescein into the anterior chamber (grade 3).* D: *Fundus photograph immediately after sham laser, in which laser spots were placed adjacent to the retinal vessels, to produce retinal injury but preserving normal retinal vasculature.* E: *The iris appears normal 12 days after sham laser.* F: *Fluorescein angiography of the iris in E demonstrates no leakage of fluorescein from the normal iris vessels (grade 0).*

the occluded veins, and fluorescein leakage and retinal edema in the posterior pole. Fluorescein angiography of a sham lasered eye demonstrated staining of the laser lesions with normal retinal vasculature. The severity of the laser injury is identical, and the different fundus appearance is due to disruption of the venous blood flow in the retina following laser vein occlusion. The eyes with laser vein occlusion and ischemic retina developed progressive iris neovascularization characterized by marked leakage of fluorescein from tortuous vessels (grade 3; Figure 2, B and C). Sham lasered eyes did not develop iris neovascularization (grade 0; Figure 2, E and F), but in some cases demonstrated mild fluorescein leakage (grade 2), probably secondary to mild inflammation induced by serial aqueous sampling.

Figure 3A shows how the VEGF levels in the ischemic (laser vein occlusion) and nonischemic (sham lasered) eyes change over time. VEGF was undetectable in the aqueous of eyes with sham laser and nonischemic retina, whereas it rose to 30 pM or more in the ischemic eyes. The corrected VEGF level (ischemic minus nonischemic) increased significantly over time ($P \leq 0.001$). Iris fluorescein angiograms were graded for degree of neovascularization in the ischemic and nonischemic eyes, and similarly plotted *versus* days after retinal laser treatment in Figure 3B. In this experiment, the eyes were only followed for 13 to 15 days after laser, and iris neovascularization was still increasing in the ischemic eyes. The sham lasered nonischemic eyes developed mild leakage on angiography (corresponding to a grade of 2), but did not progress, and the corrected iris grade increased significantly during this period ($P = 0.01$). Finally, the corrected VEGF level and iris grade over time are plotted against one another in Figure 3C. Whereas

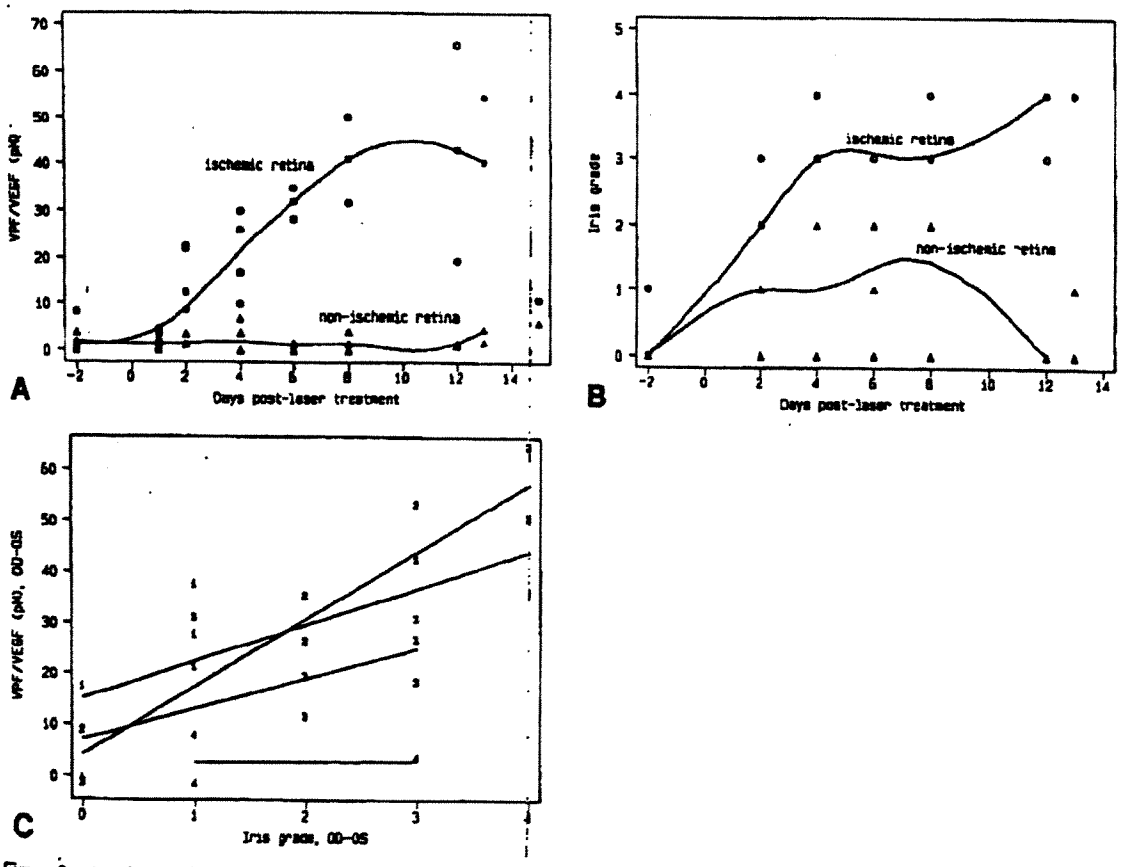

Figure 3. *Correlation of aqueous VEGF levels and iris neovascularization grade in eyes with laser vein occlusion versus sham laser.* A: *VEGF levels in the ischemic laser vein occlusion eyes (•), and nonischemic, sham lasered eyes (Δ) are compared over time as a scatterplot with best fit curves. The corrected VEGF level (ischemic minus nonischemic) increased significantly over time ($P \leq 0.0001$). One monkey died on day 5 after laser, so that data after this time point is for six eyes only.* B: *Iris grade in the same group of eyes compared over time as a scatterplot with best fit curves (ischemic •; nonischemic Δ). Two of the sham lasered eyes developed grade 2 leakage, with mild, late leakage on angiography, probably secondary to mild inflammation induced by serial aqueous sampling. The corrected iris grade increased significantly over time ($P = 0.013$).* C: *The corrected VEGF level and iris grade over time are shown, with each number corresponding to a monkey. Whereas, there is substantial variation from animal to animal, there is a statistically significant relationship between increasing VEGF level and increasing neovascularization ($P \leq 0.0001$). Monkey 4 died on day 5 after laser and was included in the analysis, although the curve from this animal clearly has a different slope. It can be postulated that there was insufficient time for much difference to develop between the two eyes (ischemic and nonischemic) in this animal.*

there is substantial variation from monkey to monkey, there is a statistically significant relationship ($P \leq 0.001$) between increasing VEGF level and increasing neovascularization. These results demonstrate that aqueous VEGF level and grade of iris neovascularization track together over time in this model of iris neovascularization.

Vitreous VEGF levels were obtained at the time of sacrifice from only a few animals throughout the experiments. In the five ischemic eyes assayed, the vitreous VEGF levels were equal to or higher than the levels measured in simultaneously obtained aqueous. This result supports the hypothesis that the VEGF is released by ischemic retina in the posterior segment of the eye, diffuses forward, and induces iris neovascularization. Vitreous levels in the nonischemic eyes were low or undetectable (data not shown).

Analysis of VEGF in Ischemic versus *Nonischemic Retinas*

To confirm that VEGF is produced by ischemic retina, the expression of VEGF mRNA was investigated by Northern analysis. Retinas were isolated from one eye with laser vein occlusion and iris neovascularization, and from one eye that received a sham laser and was without iris neovascularization. Two VEGF transcripts were identified in the ischemic retina, a distinct and high abundance band at 3.6 kb and a faint, low abundance band at approximately 3.9 kb. This pattern is in agreement with VEGF transcripts observed in other systems.[28,29] The authors have subsequently identified the monkey retina transcripts by polymerase chain reaction analysis to be the 121-kd and 165-kd forms of VEGF (Shima et al, manuscript in preparation). In contrast to the ischemic retina, VEGF mRNA was barely detectable in the nonischemic retina (Figure 4), consistent with upregulation of VEGF protein in ischemia.

Localization of VEGF mRNA in Ischemic Retina by in Situ *Hybridization*

In situ hybridization with a VEGF riboprobe was performed on ischemic and nonischemic retinas and demonstrated intense labeling of the inner nuclear layer of ischemic retina in eyes with iris neovascularization (Figure 5, A and B). In contrast, there was minimal labeling in the nonischemic retinas of eyes without iris neovascularization (Figure 5, C and D). No specific cellular labeling was seen with control sense probes and background levels were low (Figure 5, E and F). There was no definite labeling of iris tissue in either group of eyes. The retinal pigment epithelium bound both VEGF riboprobe and control sense probe, making interpretation in this cell layer difficult (data not shown).

Figure 4. *Northern blot analysis of ischemic and nonischemic retinas. VEGF Northern blot of total cellular RNA of retinas isolated from eyes with ischemic retina and iris neovascularization* (lane I), *and contralateral eyes with sham laser and nonischemic retina without iris neovascularization* (lane n) *probed for VEGF. Two VEGF transcripts were identified in the ischemic retina, a distinct and high-abundance band at 3.6 kb and a faint, low-abundance band migrating just above it at 3.9 kb.*

Discussion

Our findings demonstrate a clear temporal and spatial relationship between a secreted angiogenic factor, VEGF, and the development of heightened vascular permeability and ocular neovascularization *in vivo*. Before laser vein occlusion, the VEGF level in the aqueous was undetectable; within 2 days of laser treatment, the aqueous levels had risen, and neovascularization was detectable by 4 days. Aqueous levels of VEGF peaked in the second week after vein occlusion, coincident with the most severe neovascularization; the aqueous VEGF levels fell before regression of the neovascularization. When the aqueous VEGF levels were determined and angiography was performed every 2 to 4 days, the aqueous VEGF levels and the severity of iris neovascularization increased synchronously and proportionally. The increase in aqueous VEGF was not simply a response to laser injury inasmuch as control eyes receiving a sham laser treatment of equivalent extent did not have elevated VEGF. This is the first time, to our knowledge, that levels of an angiogenic factor have been shown to be related to the extent of neovascularization over time *in vivo*.

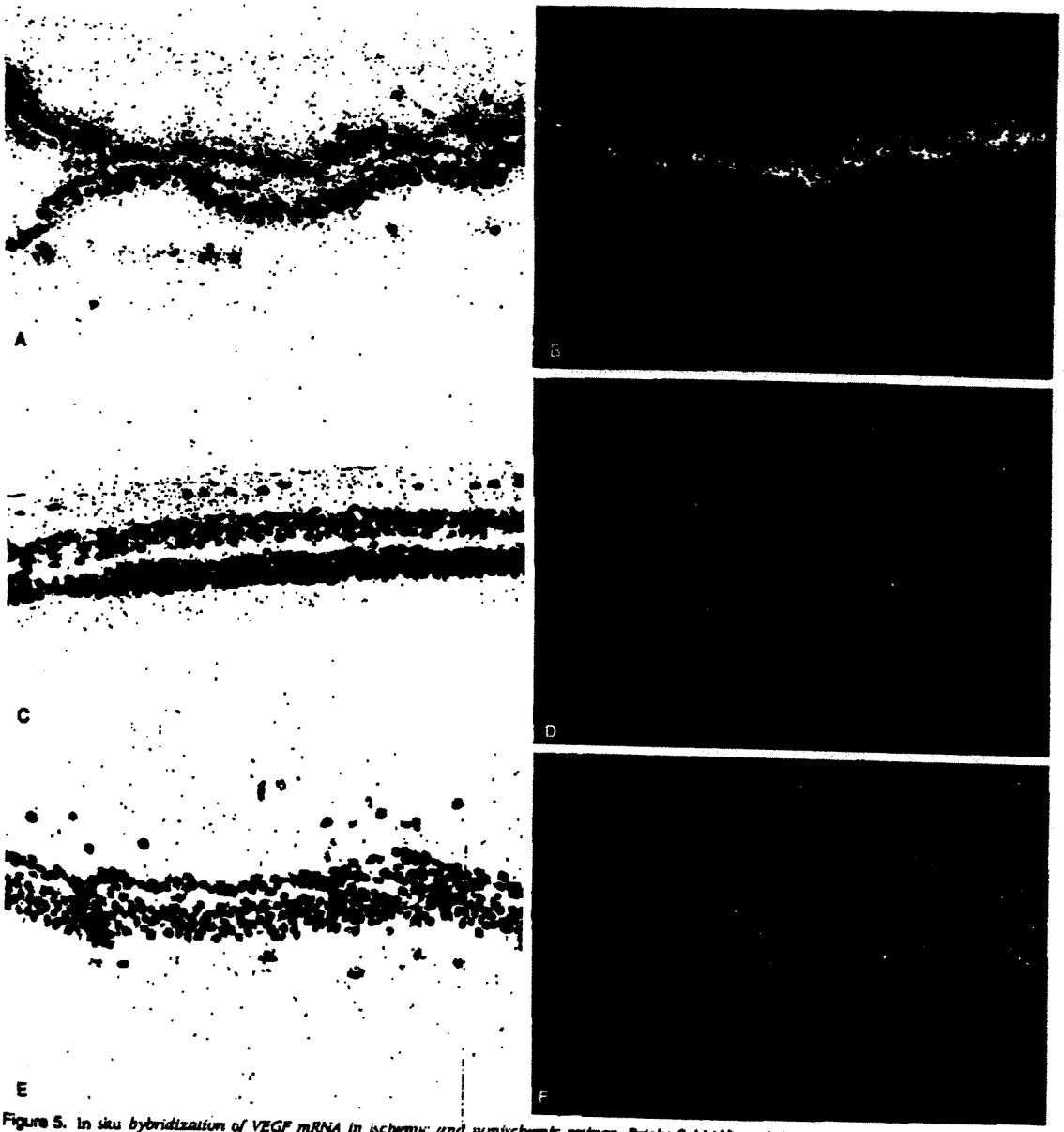

Figure 5. *In situ hybridization of VEGF mRNA in ischemic and nonischemic retinas. Bright-field* (A) *and dark-field* (B) *view of ischemic retina with a VEGF riboprobe, showing intense labeling of the inner nuclear layer. Bright-field* (C) *and dark-field* (D) *view of nonischemic retina with a VEGF riboprobe showing minimal labeling. Bright field* (E) *and dark field* (F) *of ischemic retina with a VEGF control sense riboprobe.*

The evidence for ischemic retina as the source of the increased aqueous VEGF is both indirect and direct. Serum VEGF remained undetectable through the course of the experiment, making it unlikely that VEGF was derived from the blood. Vitreous levels of VEGF were equal to or higher than aqueous levels in eyes with iris neovascularization, suggesting that the VEGF was being released from a source in the posterior segment. This is consistent with the hypothesis that VEGF is secreted from the retina into the vitreous, diffuses into the aqueous, where it induces iris neovascularization. More direct evidence implicating the retina as the source of the VEGF is provided by results of *in situ* hybridization and Northern analysis. Northern analysis of the total RNA isolated from ischemic and nonischemic retinas demonstrated a marked increased in VEGF mRNA transcripts only in the retina that was rendered ischemic by laser vein occlusion. *In situ* hybridization revealed that VEGF mRNA localized to the inner nuclear layer of the ischemic retina. Whereas the cell type expressing the VEGF mRNA could not be determined from these experiments, the

Mueller cell is a possible candidate, because its cell body is located in the inner nuclear layer, and it has cell processes that span the entire retina, filling spaces between axons and blood vessels in the nerve fiber layer. Equally likely are the mural cells (smooth muscle cells or pericytes) of the vasculature, which have been reported to synthesize VEGF.[29-31] Immunolocalization studies using cell-specific markers are underway and will identify the cell type(s) involved in VEGF expression.

The physical and metabolic changes in the retina following laser vein occlusion may be complex, but a predominant change is local hypoxia. Pournaras et al[32,33] used O_2-sensitive micro-electrodes to characterize oxygen levels in a related model of branch vein occlusion in the miniature pig. They found that the inner retina becomes hypoxic following branch retinal vein occlusion, with the lowest pO_2 measurements at a 50% retinal depth, corresponding to the interface of the inner nuclear layer and the outer plexiform layer. Our findings of increased VEGF mRNA in this layer are consistent with a hypoxia-induced upregulation of VEGF following laser vein occlusion.

Pan-retinal photocoagulation is used clinically to treat iris and retinal neovascularization that occurs secondary to diabetic retinopathy and retinal vein occlusion. In this treatment, large areas of retina are ablated using laser photocoagulation, and distant neovascularization on the optic nerve or iris subsequently regresses. Possible explanations for the efficacy of this treatment include: destruction of cells producing an angiogenic factor, attenuation of the metabolic demand, induction of an angiogenic inhibitor, and reduction of hypoxia by increasing diffusion from the choroid. Pournaras et al measured pO_2 in the pig retina after retinal vein occlusion followed by pan-retinal photocoagulation and found that the procedure reduced hypoxia in the retina.[33] Our findings suggest that neovascularization associated with retinal vein occlusion results from a hypoxia-induced upregulation and release of VEGF; thus pan-retinal photocoagulation, by reducing hypoxia, may downregulate VEGF leading to regression of neovascularization.

Many ophthalmic diseases, including proliferative diabetic retinopathy, are characterized by ischemia and neovascularization, and VEGF may play an important role in mediating these diseases. Preliminary results measuring vitreous levels of VEGF in eight diabetics and 12 control patients undergoing vitrectomy, indicate that VEGF is significantly elevated in the vitreous of patients with proliferative diabetic retinopathy.[34] If further investigations corroborate the role of VEGF in mediating neovascular eye diseases, then therapies directed at blocking the production or action of VEGF may have significant clinical application. Recent studies in tumor models using VEGF neutralizing antibodies suggest that such an approach is feasible.[35]

There is increasing evidence that VEGF and its receptors serve an important role in various angiogenesis-dependent processes. Millauer et al have demonstrated increased expression of flk-1, a high-affinity receptor for VEGF, in endothelial cells and perivascular tissue in embryonic mice compared to adult tissue.[36] High levels of VEGF mRNA have been demonstrated in the rat corpus luteum compared to the mural granulosa cells at a time corresponding to maximal angiogenesis, suggesting a physiological role for VEGF in ovulation.[30] Bovine aortic smooth muscle cells express VEGF mRNA and secrete a VEGF-like endothelial cell mitogen, and binding sites for VEGF have been demonstrated in quiescent vessels *in vivo*,[30] implying that VEGF may have a role in maintaining normal vasculature. A particularly high density of binding sites for VEGF have been found on adult rat heart valves and aorta.[30,37] In vascular regions subjected to high or turbulent flow, there may be a need for a factor to maintain the endothelial integrity.

Weindel et al found that cells cultured from acquired immune deficiency syndrome-related Kaposi's sarcoma, a vascular cell tumor, expressed more VEGF mRNA than normal human umbilical vein endothelial cells or human vascular smooth muscle cells.[29] High levels of VEGF mRNA have been demonstrated in various tumors including, human adenocarcinoma and glioblastoma, a highly vascularized tumor, in which VEGF mRNA expression was particularly high in tissue adjacent to necrotic centers, presumed to be hypoxic.[12,14,15] Thus VEGF seems to play an important role in development, in physiological maintenance of vessels, in physiological ovulation, and in pathological tumor growth. Our findings suggest an important role for VEGF in pathological ocular neovascularization, which further supports its identity as a universal angiogenic factor.

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